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GRANT NUMBER: DAMD17-94-J-4459

TITLE: A New Immunologic Method for Detection of Occult Breast Cancer

PRINCIPAL INVESTIGATOR: Dr. Rishab K. Gupta

CONTRACTING ORGANIZATION: John Wayne Institute for Cancer Treatment and Research
Santa Monica, California 90404

REPORT DATE: October 1995

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1995	Annual 1 Oct 94 - 30 Sep 95

4. TITLE AND SUBTITLE
A New Immunologic Method for Detection of Occult Breast Cancer

5. FUNDING NUMBERS
DAMD17-94-J-4459

6. AUTHOR(S)
Dr. Rishab K. Gupta

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
John Wayne Institute for Cancer Treatment and Research
Santa Monica, California 90404

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

We investigated the incidence of a 90kD subunit containing glycoprotein tumor-associated antigen (TAA)-specific immune complexes (IC) in sera of breast cancer patients and correlated it with other tumor markers - CEA and CA15-3. Sera were obtained from 106 patients with histopathologically proven breast cancer, and from 107 self-proclaimed healthy females. Serum samples were analyzed for the presence of the 90kD glycoprotein TAA-specific IC by a murine monoclonal antibody, AD1-40F4, based ELISA. The antibody, developed against an autoimmunogenic glycoprotein TAA, recognized an epitope that is different from those recognized by human polyclonal antibodies. IC captured by AD1-40F4 were realized by enzyme conjugated goat anti-human IgG. A positive test corresponded to a normalized ELISA value above 0.410 OD (mean plus 3 SD at 405nm). The incidence of 90kD glycoprotein TAA-specific IC was significantly higher ($p < 0.05$) in breast cancer patients (63%, or 67/106) than in healthy controls (2.8%, or 3/107). Comparision of the glycoprotein TAA-specific IC results in breast cancer patients with evidence of disease with results of CEA and CA15-3 revealed that the incidence of abnormal values was increased to 91%. Thus, use of the glycoprotein TAA-specific IC marker in conjunction with CEA and/or CA15-3 may prove to be more sensitive than when used alone for immunodiagnosis and immunoprognosis.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Glycoprotein, Tumor-Associated Antigen, Breast Cancer, Immune Complexes, CEA, CA15-3, ELISA, Murine Monoclonal Antibody.	20
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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*U.S.GPO:1993-0-358-779

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Rishab K. Gupta 10/25/95

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INTRODUCTION

Breast cancer is the second leading cause of death among females with cancer [1, 2]. The overall survival for breast cancer patients is considerably low, which is in part due to the fact that by the time the disease is diagnosed, it may have already metastasized [3]. A number of tumor markers have been developed for diagnosis, prognosis, and early detection of recurrence in breast cancer patients [4, 5, 6]. Most of the markers described thus far are not immunogenic in breast cancer patients.

The presence of tumor-associated antigens that are immunogenic in breast cancer patients has now been confirmed by various investigators [7, 8, 9]. Therefore, it is logical to assume that circulating immune complexes (IC) should form in vivo each time a humoral immune response is made to the antigens, and the source of the antigen is present in breast cancer patients.

Salinas et al [10] reported that immune complexes (IC) could be demonstrated in 50% sera of breast cancer patients at the time of diagnosis. Patients with metastatic disease had more IC than patients with limited disease. The IC values were correlated with other markers, e.g., carcinoembryonic antigen (CEA), and it was suggested that IC measurements might provide additional prognostic information, particularly in patients who do not have elevation of other markers [10, 11]. Subsequent reports from various investigators revealed that results of IC detection and correlation with malignant disease varied from investigator to investigator due to the antigen non-specific nature of the IC detection assays [12]. This is because the cancer patients might have had apparent and unapparent infection or autoimmune disease at the time of serum collection. These situations most likely caused some false positive IC values, and thus lowering of prognostic significance of the assay. Therefore to ascertain whether the presence of IC detected in the circulation of a cancer patient at a given point in time was due to the presence of tumor, it was necessary to use cumbersome procedures to confirm that the antigen portion of the IC was indeed the defined TAA [13]. To overcome this problem, we have isolated immune complexes from IC positive sera and after dissociation, characterized antibody and antigen components [14]. One of the antigen identified in the antigenic fraction of cancer patient's IC was similar to a heat stable glycoprotein expressed by cancer cells of various histologic types [15]. The antigen was purified from urine of a melanoma patient, because its presence was in a relatively high concentration, and was used as immunogen to develop a murine monoclonal antibody (MuMoAb), AD1-40F4, of IgM isotype [16]. Analysis by Western blot revealed that AD1-40F4 monoclonal antibody recognized a 90kD subunit of the antigen [17]. The MuMoAb showed no immunologic reactivity with human serum proteins and the epitope recognized by the monoclonal antibody resided in the protein part of the glycoprotein TAA [16]. Blocking studies revealed that the epitope on the 90kD subunit recognized by the MuMoAb was different from those recognized by the allogeneic anti-glycoprotein-TAA antibodies [18].

The present study investigates the presence of a 90kD subunit containing glycoprotein tumor-associated antigen (TAA) specific IC in the sera of breast cancer patients, and its correlation with other tumor markers, such as CEA and CA15-3.

BODY

MATERIALS AND METHODS:

In this investigation serum samples were procured from 106 women who were diagnosed to have breast cancer. The mean age of the patients was 51 years with a range of 25 to 82 years. Histopathologically, 90 patients had invasive ductal carcinoma (IDC) and 16 patients had ductal carcinoma in situ (DCIS). In addition, serum samples were procured from 107 self-proclaimed apparently healthy normal females. The age of normal controls ranged from 28 to 74 years with a mean of 45. All serum samples were stored frozen without any preservative at -35C until used.

We have developed a tumor antigen specific IC detection assay which utilizes an immobilized murine monoclonal antibody, AD1-40F4, directed to the 90kD subunit of a glycoprotein TAA. The murine monoclonal antibody, AD1-40F4, and the glycoprotein antigen were prepared as described below. Details of the assay have been described elsewhere [8]. In brief, one hundred microliters of the AD1-40F4 ascites diluted to a protein concentration of 100 ug/ml were dispensed into each of the appropriate wells of glutaraldehyde activated microtiter plates (Dynatech Laboratories, Chantilly, VA). The plates were incubated at 4C for 16 h and then washed with PBS supplemented with 0.5% Triton X-100 (PBS-TX). The washed plates were blocked with 100 ul per well of 1% bovine serum albumin (BSA) in PBS-TX at 23C for 1.0 h. Test serum samples were diluted 1:60 with PBS-TX supplemented with 1% BSA, 0.5% normal mouse serum and 0.01M ethylene diamine tetraacetic acid (EDTA). One hundred microliters of the diluted sample were dispensed into duplicate wells of the activated plates and incubated at 37C for 45 min. At the end of incubation the wells were washed with PBS-TX. One hundred microliters of alkaline phosphatase conjugated to Fab fragment of goat anti-human IgG (Sigma Chemical Company, Saint Louis, MO) at 1:500 dilution were added to each test and control wells of the plate. The plates were incubated at 37C for 45 min and washed with PBS-TX. Each well of the plate then received 200 ul of p-nitrophenyl phosphate (1.0 mg/ml) in 10% diethanolamine buffer as the substrate and the plates were incubated in the dark for 1.0 h at 23C. The absorbance was read at 405_{nm}. Each sample was tested in duplicate with positive and negative controls and blanked individually in the same microtiter plate. Each test plate also included controls for non-specific protein binding and binding of conjugate to the immobilized murine monoclonal (capturing) antibody. The net optical densities of the control samples were used to generate a correction factor to normalize the net optical density of the test samples analyzed on that particular test plate. If the correction factor for a test plate fell outside the range from 0.8 to 1.2, the assay was considered invalid. The upper limit of normal for the glycoprotein TAA marker was set at 0.410 (mean + 3 SD ELISA values of 59 normal sera determined from previous studies).

The glycoprotein TAA which is expressed by 82% (18/22) carcinomas, was purified as described elsewhere [18] from a 24 h urine sample of a melanoma patient. Urine from melanoma patient (Je 8504) was used because the glycoprotein TAA is expressed by solid tumors of various histologic types and this patient's urine had relatively high antigenic activity [18]. Briefly, the 24 h urine samples were collected and filtered through a Whatman no. 1 filter-paper

(Whatman International, Maidstone, England) to remove all sediments. The clarified urine was concentrated 100-fold using an Amicon hollow-fiber concentrator equipped with an H1P10-8 cartridge (Amicon Corp., Beverly, Mass.). Concentrated material was passed through a Sephacryl S-200 column (Pharmacia LKB, Piscataway, N.J.) using 0.025 M phosphate buffered saline supplemented with 0.02% sodium azide as eluent at a flow rate of 25ml/h. Fractions under each peak observed at 280_{nm} were pooled separately, concentrated and tested for antigenic activity using an allogeneic double-determinant enzyme-linked immunosorbent assay as described previously [16]. The antigenic pool was quantitatively absorbed with immobilized rabbit anti-human Ig antibodies until free of detectable human IgG in an enzyme immunoassay [17]. The purified antigenic pool was used to develop murine monoclonal antibodies.

The glycoprotein TAA prepared as described above was used as an immunogen to develop a murine IgM monoclonal antibody, AD1-40F4, with specificity to the antigen according to the procedures described by Kohler and Millstein [19]. The monoclonal antibody did not exhibit any reactivity with pooled human IgM, pooled human IgG, ferritin, B2-microglobulin, B2-glycoprotein, apolipoprotein B, apolipoprotein CII, apolipoprotein CIII, or human serum albumin. Western blot analysis revealed that the AD1-40F4 antibody recognized the 90kD subunit of the glycoprotein antigen [16]. After specificity analysis, the murine monoclonal antibody was mass produced as ascites in BALB/c mice, and used as the source of antibody to develop the 90kD-TAA-specific IC detection assay as previously described.

CEA measurements on 68 of the 106 serum samples were performed by Dianon Systems, Inc., Stratford, Connecticut, using Abbott CEA-EIA procedures that followed manufacturer's instructions. Results were expressed as ng CEA/ml. A value of greater than 2.5 ng CEA/ml was considered positive.

CA15-3 was measured by the radioimmunoassay kit (CA15-3 RIA) commercially available from Centocor, Malvern, PA. The RIA was performed according to the manufacturer's instructions by the Dianon Systems, Inc. Results were expressed as CA15-3 units/ml (U/ml). A value of greater than 30 U of CA15-3/ml was considered positive.

Fisher's exact test as implemented by the Instat Biostatistics program from the GraphPad Software, Inc., San Diego, Ca, was used to determine statistically significant differences among the 90kD glycoprotein TAA-specific IC assay values of normal and breast cancer patients, and for comparision between 90kD TAA-specific results and other tumor marker results. All comparisions were two-tailed and a p value of less than 0.05 was considered statistically significant.

RESULTS:

A polyclonal anti-glycoprotein TAA antibody purified from a baboon antiserum that was raised against the glycoprotein-TAA [20], was used to generate IC in vitro. For this purpose the baboon polyclonal antibodies were mixed with the purified glycoprotein TAA in different protein proportions. After incubation at 37C for 30 min, the mixtures were tested to determine

if a positive signal was generated in the AD1-40F4 murine monoclonal antibody capture assay. Goat anti-human IgG conjugated to alkaline phosphatase was used as the signal developer. We have previously documented that the goat anti-human IgG enzyme conjugate reacts equally well with the baboon IgG. Table I denotes that binding of the enzyme conjugate was the highest when the immobilized AD1-40F4 murine monoclonal antibody was incubated and reacted with the mixture of purified glycoprotein TAA and purified polyclonal baboon anti-TAA IgG. Furthermore, this signal was consistently high over a wide range of antibody (baboon anti-TAA) to antigen (glycoprotein TAA) at protein concentration ratios (3:1 to 90:1). Neither of the two immune reactants (antigen or antibody) alone or pre-immune baboon IgG or human serum albumin exhibited a signal greater than 0.200 O.D. at 405_{nm}. These data confirmed our previous [16] observations that the AD1-40F4 murine monoclonal antibody had no significant reactivity with baboon IgG and human serum albumin, and that the enzyme conjugate had no affinity (specific or non-specific) with either the murine monoclonal antibody or with the glycoprotein TAA. These results clearly denote that the AD1-40F4 murine monoclonal antibody captured baboon anti-glycoprotein TAA IgG via the glycoprotein TAA only when the IgG antibody was in the form of glycoprotein TAA-specifc immune complexes.

Reproducibility studies to assess the assay variability using a serum from breast cancer patients in ten replicates revealed that inter-assay variations ranged from 0.806 to 1.311 ELISA value with a mean of 1.010, standard deviation of 0.139 and coefficient of variation of 13.7%. These values for intra-assay variations ranged from 0.849 to 1.214 with a mean of 1.007, standard deviation of 0.105 and coefficient of variation of 10.4%.

Initially we analyzed serum samples from self-proclaimed healthy normals and breast cancer patients. Figure 1 illustrates the distribution of 90kD TAA-specific IC ELISA values of sera from 107 healthy normal females and from 106 breast cancer patients. In this experiment, the procurement of the sera from breast cancer patients was random, i.e., no criterian with respect to pre- or post-surgery, evidence or no evidence of disease, etc., was used in selecting the serum samples. Comparative analysis of the data in normal and breast cancer group revealed that the normalized ELISA value (mean $\pm$ SD) of the normal control sera (0.212 ± 0.088) was significantly ($p < 0.05$) lower than that of breast cancer patients (0.570 ± 0.438). Furthermore, when an ELISA value of 0.41 or greater was considered positive for the presence of the TAA-specific IC, the incidence of the glycoprotein TAA-specific IC was significantly ($p < 0.05$) greater in the breast cancer group (67/106, 63%) than the normal group (3/107, 2.8%).

While the incidence of 90kD TAA-specific IC in the normal group was not affected by age, it was significantly higher ($p < 0.05$) in breast cancer patients that were over 60 years old (88%, 23/26) compared to those that were under 60 years old (55%, 44/80).

Of the 90 IDC breast cancer patients, 58 (64%) were positive for the glycoprotein TAA-IC; whereas, this incidence was lower 56% (9/16) in DCIS breast cancer patients (Table II). The lower incidence in DCIS patients may be due to the non-aggressive nature of the ductal carcinoma in situ.

CEA and CA15-3 have been considered useful tumor markers in the prognosis and monitoring of breast cancer patients. We compared the glycoprotein TAA-specific IC results using 68 serum samples of breast cancer patients selected on the basis of the presence of the disease. The two tumor markers, CEA and CA15-3, are not known to be immunogenic in cancer patients. Of the 68 serum samples 55 (80.9%) were positive for the glycoprotein TAA-specific IC, 16 (23.5%) were positive for CEA, and 23 (33.8%) were positive for CA15-3. Despite higher incidence of glycoprotein TAA-specific IC than CEA or CA15-3, it was observed that some serum samples that were positive for CEA or CA15-3 were not necessarily positive for the glycoprotein TAA-IC.

As shown in Table III, statistical evaluation of the data by Fisher's exact test revealed that there was no significant associations between the glycoprotein TAA and CEA or CA15-3 ($p > 0.05$). However, when either of the three or all of the three positive markers were taken into consideration, the incidence of positivity increase from 80.9% to 91% (Table IV).

DISCUSSION:

Serological tumor markers are considered to be useful in the early detection and monitoring of metastases for early and effective treatment to increase the duration of disease free and/or overall survival [21, 22]. However, with the exception of CA15-3, recent reports have questioned the value of many of these sensitive markers both in diagnosis of systemic disease and in assessing response to therapy [23, 24]. In this investigation, we have analyzed serum samples from breast cancer patients to determine the usefulness of an antigen specific immune complex detection assay. This marker differs from the existing tumor markers in that it determines the presence of a glycoprotein TAA which is immunogenic in patients and circulates in the form of immune complexes in the blood. The detection assay can be considered as a form of double-antibody sandwich ELISA in which the immune complexes present in the test sample are captured by an immobilized murine monoclonal antibody, AD1-40F4. This monoclonal antibody was specifically developed using the purified glycoprotein TAA defined by autologous and allogeneic antibodies, and recognizes an epitope different from those recognized by the autologous antibodies.

There is sufficient evidence in literature to suggest that estimation of immune complex levels or their fluctuations during the course of malignant disease might predict the outcome of the disease [10]. However, unlike other tumor markers, one of the drawbacks which inhibited the application of this technology in a clinical setting has been the use of antigen non-specific assays for the detection of immune complexes. The use of antigen-nonspecific assays resulted in inconsistent results, because some of the material detected by these assays were characterized to be aggregated IgG, reaction products of denatured self-proteins, polyamines, or bacterial lipopolysaccharides [25]. In general, immune complexes detected in sera of cancer patients have been characterized with respect to their size and the presence of anti-Ig and anti-tumor antibodies, and tumor or other antigens. A number of methods have been used to isolate and characterize the antibody and antigen components of the immune complexes [26]. However, manipulations of the *in vivo* formed immune complexes are prone to introduce artifacts for

characterization in subsequent studies; thus, providing inaccurate results. Therefore, development of an assay such as the one used in this investgation which detects antigen-specific immune complexes without any pre-treatment or manipulation of the test sample represents a significant and major advancement in the area of immunodiagnosis of human cancer.

It can be argued that immune complexes present in circulation may be composed of either IgG or IgM antibodies or both; however, anti-tumor antibodies of IgG type to macromolecular antigens are more prevalent [27], and the 90kD glycoprotein TAA-specific IC assay can be modified to detect immune complexes containing IgM antibody by using anti-human IgM conjugate. We feel that the success of the 90kD glycoprotein TAA-IC assay for the detection of cancer is for the following reasons. The immunogenic tumor antigens shed into circulation by growing tumor cells are in small quantities and are neutralized by the humoral antibodies [28]. Therefore, the results of any sensitive methods applied to detect free antigens in serum or plasma are generally negative; however, detection of human antibody (immunoglobulin) molecules via the antigen captured by the immobilized MuMoAb gives an amplification effect. This is because the unreduced antigen is a complex of at least four different subunits [18], each of which is immunogenic in the cancer host and thus can bear multiple in vivo reacted immunoglobulin molecules. Furthermore, glycoprotein TAA being immunogenic in cancer patients should circulate in the bloodstream in the form of immune complexes, particularly at the time when the source of the antigen (tumor) is present only in small amounts.

The serum level of 90kD glycoprotein TAA, as assessed in the form of immune complexes, was uniformly low in the control group of 107 apparently healthy females. The values (0.212 ± 0.088 OD_{405nm}) observed in this investigation were comparable to those reported earlier (0.249 ± 0.080 OD_{405nm}) from our laboratory where the control group was comprised of 250 normal males and females [8]. Furthermore, the incidence of positive values were comparable (2.8% vs 3.2%). In this investigations where serum samples were obtained from patients with a history of breast cancer, elevated serum levels of 90kD glycoprotein TAA were observed at a frequency of about 63%. These results confirm and significantly expand the initial observation that the 90kD glycoprotein TAA marker could be detected in greater proportion of breast cancer patients as well [29]. It is of particular interest to note that the incidence of 90kD glycoprotein TAA in breast cancer patients was affected by age. It has been reported that the mortality rate in younger breast cancer patients is significantly lower in contrast to older breast cancer patients [30, 31]. Patients with an age of greater than 60 years showed significantly greater incidence than those patients who were younger than 60 years. It would appear as if the tumors of younger women do not express this antigen or the tumor cells do not release it into circulation. Is it due to dormancy or different metabolic turnover rates of surface molecules? We are currently in the process of correlating the expression of the glycoprotein TAA with the level of expression of estrogen/progesterone receptors by the breast cancer cells. These hormones have been reported to modulate the expression of surface macromolecules, e.g. cerebellar responses to amino acid neurotransmitters (32).

It is obvious that the 90kD glycoprotein TAA-IC detection assay described here is not 100% accurate at this time in identifying breast cancer sera. Furthermore, as low it may be,

certain proportion of sera from normal controls was positive for the marker. The presence of 90kD glycoprotein TAA in normal population is unexplainable at this time; however, it may be possible that these apparently healthy individuals had occult neoplasm at the time of serum sampling. This possibility of existence of occult disease is difficult to prove or disprove until longitudnal follow-up of these individuals is performed.

Despite the fact that we have not reached 90% or greater incidence for the presence of 90kD glycoprotein TAA-specific IC in breast cancer patients, the results obtained thus far are highly encouraging. These investigations have discovered unique approaches to immunodiagnosis of breast cancer via detecting an immunogenic tumor antigen in circulation, its specificity can be significantly enhanced by incorporating other existing tumor markers, such as CEA [33] and CA15-3 [34-36]. Extensive evaluation of CEA in combination with other tumor markers or alone for clinical correlations with the clinical course of breast cancer patients has resulted in conflicting reports [37-40]. In fact, it has been suggested that CA15-3 correlated with the stage of disease and in metastatic patients with the response to treatment [41-46]. Use of this novel marker (tumor antigen-specific immune complexes) in conjunction with CEA and\or CA15-3 may prove to be more sensitive than when used alone for immunodiagnosis and immunoprognosis of breast cancer.

CONCLUSION

The incidence of the glycoprotein tumor-associated antigen (TAA)-specific immune complexes in breast cancer patients was 63%(67/106), as indicated by the normalized ELISA value above 0.410 OD_{405nm}. On the contrary, only 3 (2.8%) of 107 apparently healthy controls had positive ELISA value ($p < 0.05$). Comparison of the glycoprotein TAA-specific IC results in breast cancer patients with evidence of disease with the results of CEA and CA15-3 revealed that the incidence of abnormal values was increased to 91%. Thus, use of the glycoprotein TAA specific-IC marker in conjunction with CEA and/or CA15-3 may prove to be more sensitive than when used alone for immunodiagnosis and immunoprognosis. To further determine the clinical utility of this marker, we will continue to analyze sequential serum samples from breast cancer patients to see if the marker positive patients at the time of no evidence of disease develop recurrent diesease on follow-up. Such analyses will also provide information on the lead time to recurrence which would be useful to oncologists in the management of the disease. In addition, we are continuing to search for additional tumor associated antigens that are immunogenic in breast cancer patients.

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TABLE I. Detection of purified glycoprotein TAA after mixing with purified baboon polyclonal IgG antibody by the murine monoclonal antibody, AD1-40F4, capture assay.

Test material	Absorbance at 405_{nm}
Immobilized murine monoclonal antibody (control)[a]	0.042
+ baboon anti-glycoprotein TAA (44 ug purified IgG/ml)	0.138
+ glycoprotein TAA (15 ug protein/ml)	0.068
+ human serum albumin (20 ug/ml)	0.162
+ pre-immune baboon IgG (50 ug/ml)	0.129
+ mixture of baboon anti-TAA IgG (44 ug/ml) and:	
glycoprotein TAA (15 ug/ml)	0.686
glycoprotein TAA (5 ug/ml)	0.869
glycoprotein TAA (1.6 ug/ml)	0.714
glycoprotein TAA (0.53 ug/ml)	0.753
human serum albumin (20 ug/ml)[b]	0.153
human serum albumin (10 ug/ml)[b]	0.218
human serum albumin (5 ug/ml)[b]	0.176
+ mixture of pre-immune baboon IgG (50 ug/ml) and:	
glycoprotein TAA (15 ug/ml)	0.188

[a]Anti-90kD glycoprotein TAA murine monoclonal antibody in the form of ascites (100 ug protein per ml) was immobilized to the wells of glutaraldehyde-activated microtiter plates.

[b]Human serum albumin was used as control to determine the effect of nonspecific protein control.

TABLE II. Incidence and level of 90kD glycoprotein TAA-specifc IC in two different histologic type of breast carcinoma.

Histologic type	Total number	Number positive	Percent positive	ELISA values		
				Range	Mean	SD
Invasive ductal CA	90	58	64	0.000-2.038	0.453	0.365
Ductal CA in situ	16	9	56	0.046-1.357	0.412	0.314

TABLE III. Association between serum glycoprotein TAA-IC and CEA or CA15-3.

		Glycoprotein TAA-IC		
		Positive[*]	*Negative*	*Total*
CEA	Positive[**]	13	3	16
	Negative	42	10	52
	Total	55	13	68
		$p > 0.05$		
CA15-3	Positive[***]	18	5	23
	Negative	37	8	45
	Total	55	13	68
		$p > 0.05$		

[*] An ELISA value of greater than 0.41 OD at 405_{nm} was considered positive.

[**] A value of greater than 2.5 ng CEA/ml was considered positive.

[***] A value greater than 30 U of CA15-3/ml was considered positive.

TABLE IV. Incidence of positivity for glycoprotein TAA-IC, CEA and CA15-3 or their combination in sera from breast cancer patients.

Marker (Alone or in combination)	(n = 68)	
	Number positive	Percent positive
Glycoprotein TAA-IC only	55	81
CEA only	16	24
CA15-3 only	23	34
CEA or CA15-3	30	44
TAA-IC or CEA	58	85
TAA-IC or CA15-3	60	88
TAA-IC or CEA or CA15-3	62	91

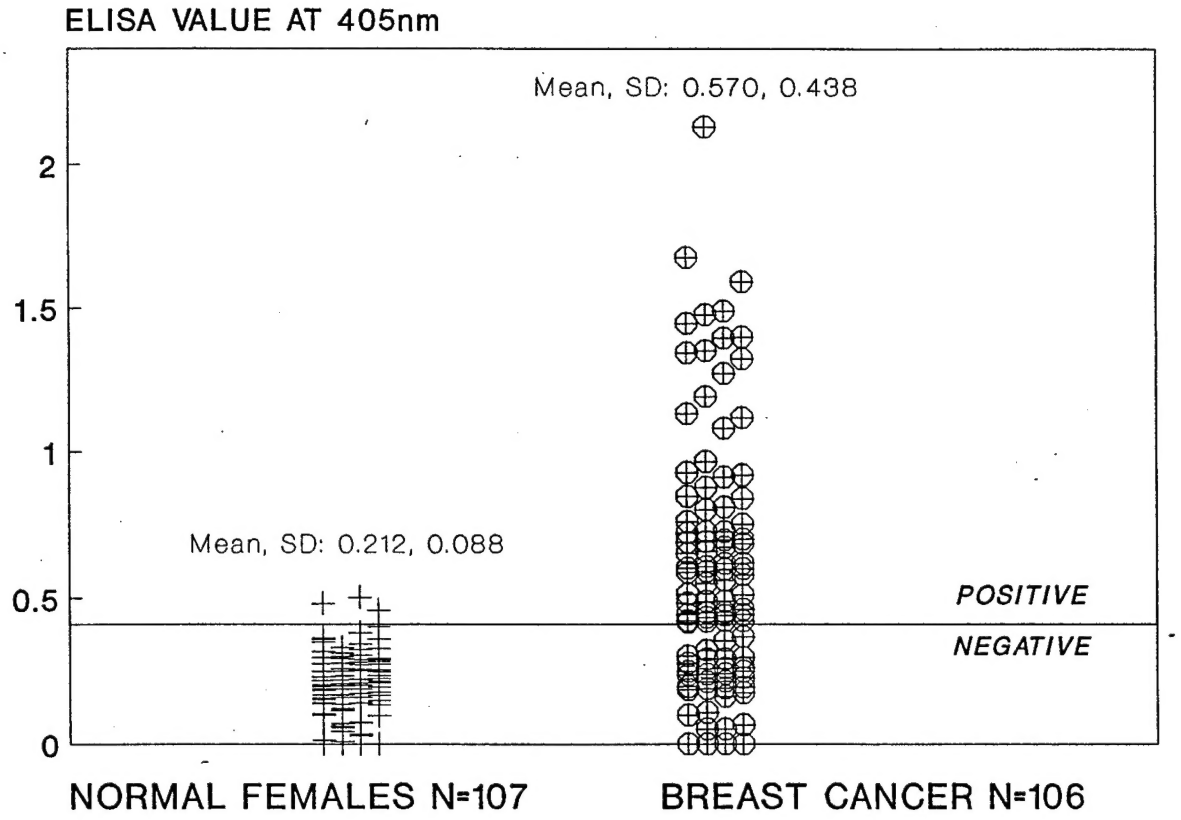

Figure 1. Incidence and distribution of 90kD glycoprotein-TAA-specific IC in sera of normal controls and randomly selected breast cancer patients. Horizontal solid line denotes the positive cut-off level.